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Fecal calprotectin: beyond intestinal organic diseases

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Running title: FC beyond organic diseases

Abstract

Fecal calprotectin (FC) is a calcium-binding protein with antimicrobial, immunomodulatory and antiproliferative properties that is mainly found in the cytoplasm of neutrophil granulocytes. During the last decades, FC became an increasingly useful tool both for gastroenterologists and for general practitioners for distinguishing inflammatory bowel disease (IBD) from irritable bowel syndrome. FC correlates with clinical scoring systems and endoscopic lesions in IBD and is considered a reliable biomarker for the prediction of clinical relapse or remission. However, FC elevation could be observed also in other gastrointestinal pathological conditions including infective colitis, microscopic colitis, eosinophilic colitis, adenomas and colorectal cancer. In addition, there are several non-pathological conditions that can lead to altered FC values.

In this review, we aimed to point out individual, environmental and method-related factors that can affect FC measurement and thus its clinical interpretation.

Key words: Biomarkers; Fecal calprotectin; Inflammatory bowel diseases.

Calprotectin is a 36.5 KDa calcium- and zinc-binding protein that belongs to the S-100 protein family.¹ Calprotectin is mainly found in neutrophil granulocytes accounting for up to 60% of the cytosolic proteins but also in monocytes, macrophages and epithelial cells. Calprotectin has several biological properties including antimicrobial, immunomodulatory and antiproliferative activities, and it is released during cell activation (active release) or cell death (passive release).^{2,3} The accumulation of neutrophils in the mucosa, a feature of inflammatory flares, results in the release of fecal calprotectin (FC) in the stools, where it can be easily measured. In particular, the presence of calprotectin in feces quantitatively relates to neutrophil migration toward the gastrointestinal (GI) tract; thus, it represents a useful marker of intestinal inflammation also because unlike other systemic inflammatory markers, its levels seem to be unaffected by causes of inflammation other than intestinal ones.

Until 1990, calprotectin was known as leucocyte protein 1 (L1); afterwards, Steinbakk *et al* observed that the calcium binding L1 protein was able to inhibit the growth of blood culture isolates of *Candida spp* and several gram-positive and -negative bacteria.⁴ Due to the biological activity of L1, the name calprotectin was proposed to describe this antimicrobial protein with calcium binding properties.⁴ Subsequently, Roseth *et al* described a method for the extraction and the quantification of calprotectin in feces by enzyme immunoassay, observing that FC was stable in stool for 7 days at room temperature and it was resistant to bacterial proteolytic degradation;⁵ even more, FC was able to accurately reflect granulocyte migration through the gut wall in patients with inflammatory bowel disease (IBD).⁶ Before these discoveries, fecal excretion of indium-111-labelled neutrophilic granulocytes was considered the gold standard of disease activity, but its complexity and high cost and the exposure of patients to ionizing irradiation had limited the use of this technique.⁶

Since 2000, numerous studies have been published showing evidence regarding FC usefulness for the detection and monitoring of a several GI disorders, especially IBD: a clear relationship has been demonstrated between the magnitude of calprotectin elevation and the extent

of intestinal inflammation⁷⁻⁹ Indeed, in clinical practice differentiating patients with organic diseases from those with functional disorders (i.e. irritable bowel syndrome [IBS]) may be difficult because of a wide spectrum of overlapping symptoms including abdominal pain, bloating, diarrhea, constipation or altered bowel habit.¹⁰⁻¹² However, it should be noted that elevated FC levels could be found not only in IBD but also in other GI pathological conditions including infective colitis, microscopic colitis, eosinophilic colitis, adenomas and colorectal cancer.¹³⁻¹⁷ FC showed high correlation with bacteriologically positive infectious diarrhoea, with sensitivity and specificity being 83% and 87% respectively.¹⁸ The high levels of FC in active collagenous colitis (CC) patients can be explained by the fact that calprotectin is present not only in neutrophils but also in monocytes and macrophages; nevertheless, the use of FC as an inflammatory marker for CC is not recommended for lack of universal evidence.¹⁹ Tursi *et al* showed that FC were significantly increased in 16 participants with acute uncomplicated diverticulitis and in 16 participants with symptomatic uncomplicated diverticular disease (SUDD), compared with 16 patients with asymptomatic diverticulosis; FC decreased to normal values after treatment, both in acute uncomplicated diverticulitis and in SUDD.²⁰

In addition, there are several non-pathological conditions, including individual, environmental and method-related factors, that can lead to altered FC values and in turn, that can mislead the clinical evaluation (Table I).

Fecal calprotectin cut-off in clinical practice

Currently, a FC result below 50 $\mu\text{g/g}$ is considered normal. According to a recent meta-analysis, FC <50 $\mu\text{g/g}$ gave a pooled sensitivity (Se) of 93% and specificity (Sp) of 94% for distinguishing between IBD and IBS in adults.²¹ In a primary care setting, Pavlidis *et al* reported that among 962 patients (18-45 years) with persistent GI symptoms, 686 (71%) patients had negative (<50 $\mu\text{g/g}$) FC results whereas 276 (29%) tested positive (>50 $\mu\text{g/g}$).²² This cut-off showed Se=82% and Sp=77% for organic diseases, with a negative predictive value (NPV) and positive

predictive value (PPV) of 98% and 28%, respectively.²³ So far, a cut-off of 50 µg/g could be suggested in order to rule out patients with IBS.

Conversely, in a secondary/tertiary care setting higher cut-off values of FC may be recommended.²⁰ Among 68 patients referred to our outpatient clinic for the first time and reporting abdominal pain and/or altered bowel habits lasting at least 4 weeks, we showed that a cut-off of 150 µg/g was able to classify correctly 90% of IBS cases and 88% of IBD cases with an area under the curve (AUC) of 0.931, indicating an excellent diagnostic accuracy; however the performance was lower for the discrimination between functional and organic diseases (AUC=0.811).¹⁴ In addition, Dhaliwal *et al* showed Se=90% in determining IBD remission (AUC=0.93) by using a cut-off less than 250 µg/g.²⁴ In Crohn disease, FC levels >250 µg/g indicated the presence of large ulcers with a sensitivity of 60.4% and a specificity of 79.5% (PPV=78.4%, NPV=62.0%).²⁵

Individual and environmental factors affecting fecal calprotectin levels

Apart from intestinal organic diseases, several individual and environmental factors are associated to increased FC values. Among demographic features, it has been reported that age less than 4 years is negatively correlated with FC levels.²⁶ Song *et al* investigated FC levels in a cohort of healthy children aged between 6 months and 4 years and found that the 95th percentile of FC values was 135 µg/g in 7-12 months group, 65 µg/g in 13-18 months group, 55 µg/g in 19-24 months group, 40 µg/g in 25-30 months group, 21 µg/g in 31-36 months group, and 12 µg/g in 37-48 months group.²⁷ Oord *et al* compared FC levels of 75 healthy children (from 1 month to 4 years of age) with those of 157 children who have had a FC analysis performed for diagnostic purposes and, on the basis of the results observed, proposed three different cut-offs according to the different age groups: 538 µg/g (from 1 to 6 months), 214 µg/g (from 6 months to 3 years) and 75 µg/g (from 3 to 4 years).²⁸ However, it is noteworthy that also older age is associated with increased FC levels.²⁹ Probably, the decline in cellular and humoral immunity associated to increased age may

affect the maintenance of the gut mucosal immune integrity causing an increased inflammatory response towards different antigenic stimuli.³⁰

Elevated FC levels have been reported in individuals with increased body mass index (BMI).³¹⁻³³ Interestingly, it has been showed that subjects with BMI > 25 Kg/m² and FC > 50 µg/g undergoing a weight loss program experienced a reduction in FC levels.³⁴ Moreover, obesity has been associated to both higher FC levels and fecal microbiota composition: obese subjects show a microbiota characterized by diminished bacterial diversity, a decreased ratio of Bacterioides to Firmicutes and higher FC levels.³⁵ Since Paneth cells play a key role in the maintenance of the GI barrier,³⁶ the diminished levels of Paneth cell derived antimicrobial proteins observed in obese subjects may partly explain the local intestinal inflammation.³⁷ Finally, in a recent study investigating the potential relationship between lifestyle factors and colorectal cancer risk, authors observed that increased age, reduced physical activity and decreased fiber intake were significantly and independently associated to higher FC levels.²⁹ Also, dietary supplements such as zinc, vitamin D, fatty acids, and several probiotics can affect FC levels.³⁸

Among confounding factors, the use of non-steroidal anti-inflammatory drugs (NSAID) and proton pump inhibitors (PPI) have been associated with FC elevation. NSAID-induced enteropathy is characterized by an increased influx of neutrophils to the intestinal mucosa and subsequent excretion into the bowel lumen, consequently leading to increased FC levels.^{39,40} Less clear is the mechanism causing FC elevation following PPI use. Poullis *et al* found that patients taking PPI had significantly elevated FC levels compared to those not on PPI (78 µg/g vs. 31 µg/g, p<0.001).⁴¹ Moreover, the effect of PPI appeared to be independent from underlying dyspepsia; as authors reported, in patients not taking PPI, FC of those with no dyspepsia was not significantly different compared to FC of dyspeptic patients (30 µg/g vs. 31 µg/g, p>0.05).⁴¹ Based on this findings, discontinuation of PPI prior to FC testing may be suggested in order to increase the specificity for detecting organic GI disorders.

Methods for fecal calprotectin assessment

Before testing, FC needs to be extracted from stool. The “gold standard” is considered the manual weighing approach through which a portion of 50-100 mg of homogenized faecal material is diluted to achieve a weight-to-volume ratio of 1:50 using extraction buffer.^{5,42} Despite its reliability, this method is time consuming and consequently unsuitable for routine use. For this reason, different commercially available extraction devices have been developed, making pre-analytical phase easier and faster. These extraction systems utilize a fixed volume of fecal material to equate a defined weight of stool and currently, completely replaced the manual extraction method.

Regarding analytical phase, the most diffused method for FC measurement is the enzyme-linked immunosorbent assay (ELISA) (Table II). This method employs a primary antibody, either monoclonal or polyclonal, to detect FC and a secondary enzyme-linked antibody that binds the primary immuno-complex. Following the formation of the “sandwich”, the enzyme converts the substrate generating a color; absorbance is spectrophotometrically read and converted in concentration based on a standard curve.⁴³ The main advantages of this method are the analytical accuracy and the possibility to test simultaneously up to 80 samples (40, if tested in duplicate) in approximately 2 hours.

Based on the same principle, novel chemiluminescent enzyme immunoassays (CLEIA) have been developed.⁴⁴ Compared to traditional ELISA, CLEIA-based methods minimize waste of reagents and are fully automated, thereby reducing the workload for the laboratory personnel. In addition, CLEIA methods offer rapid turnaround times and are less demanding in technical expertise.

During the last decade, novel rapid lateral-flow immunochromatographic methods for FC quantitation have been developed.⁴⁵⁻⁴⁷ In contrast to ELISA test, that are mostly confined to the medical laboratories, point-of-care tests (POCTs) are simple medical tests that can be performed at the bedside since not require specific and dedicated instrumentation. In addition, ELISA have long

turnaround times, making POCTs more cost-effective. However, it is unclear what could be the added value of POCTs over sending stool specimens to a laboratory for the ELISA testing, considering that management and referral decisions for non-acute GI conditions are unlikely to need immediate answers from a FC test.

More recently, a novel particle enhanced turbidimetric immunoassay (PETIA) for FC quantitation have been proposed as a less expensive and a less labor intensive alternative method to traditional ELISA.⁴⁸ Turbidimetry measure the loss of intensity of transmitted light due to the scattering effect of particles in a solution.⁴⁹ The peculiarity of PETIA method is that can be applied on standard chemistry analyzers.

Pre-analytical and analytical issues

Concerning pre-analytical phase, potential source of variability in FC measurement is represented by characteristics of stool, within-day sampling time and extraction method.⁵⁰ Lasson *et al* observed, in patients with active ulcerative colitis, that FC levels correlated significantly with both the time between bowel movements ($r=0.50$, $p=0.013$) and with the stool consistency ($r=0.68$, $p=0.001$).⁵¹ However, the latter feature could be a consequence of the disease activity. In fact, it has been reported in patients without intestinal organic disease that looser stool consistency was associated to lower FC levels,²⁹ probably due to a sample dilution effect. In addition, looser stool consistency affects sample recovery thus leading to unreliable FC results.

Devices used for sampling and extraction can affect FC results. Whitehead *et al* showed that extraction of faecal samples with three different commercial devices (Roche, ScheBo and Immunodiagnostik) led to an under-recovery of FC in comparison with the manual extraction procedure (overall mean bias from -28.1% to -7.8%).⁵² In addition, under sampling was particularly evident with watery stool.⁵²

Concerning methods of FC quantitation, several studies showed a significant degree of variability between assays. From a comparison of 3 ELISA methods emerged a significant assay

discordance: Buhlmann-fCal[®] ELISA assay tended to give higher FC results (up to 3.8 fold) than the IDK[®] Calprotectin ELISA and Calprest[®] Eurospital assays.⁵² Labaere *et al* determined calprotectin by means of three rapid immunochromatographic tests (Quantum Blue[®] Bulhmann, Calfast[®] Eurospital, CerTest[®] Biotec) two ELISA assays (Calprest[®] Eurospital and CalproLab[™] Calprotectin ELISA), and one automated fluoroimmunoassay (EliA[®] Calprotectin –Phadia); despite all methods correlated significantly, slopes and intercepts differed extensively, with up to 5-fold quantitative differences between assays.⁵³ Delefortrie *et al* reported higher FC levels measured using the Quantum Blue[®] Bulhmann when compared to DiaSorin Liaison[®] Calprotectin, independently of the extraction method used.⁵⁴

Similarly, we performed a comparison of two ELISA assays (Calprest[®] Eurospital and Buhlmann-fCal[®] ELISA) on a total of 60 fecal samples collected from 23 outpatients with functional intestinal disorders and 37 with organic diseases (unpublished data). The two assays showed both high precision (Pearson $\rho=0.9298$) and high accuracy (Bias correction factor $C_b=0.9199$), with an overall concordance correlation coefficient of 0.8553. However, Buhlmann-fCal[®] ELISA assay showed an overall overestimation of FC levels compared to Calprest[®] Eurospital assays (Figure 1).

Conclusions

FC is a reliable non-invasive tool for assessing intestinal inflammation and plays a relevant role in clinical practice. However, clinicians must be aware that FC levels could be affected by several non-pathological conditions, including individual, environmental and method-related factors. Thus, the following key messages may be highlighted for clinicians.

Since age, BMI, lifestyle and diet could affect FC values, the laboratory results must always be interpreted according to the clinical context and in case of inconsistent results, FC measurement should be repeated.

Different cut-off values may be adopted; in primary care setting, an FC result $<50 \mu\text{g/g}$ is accurate to rule out an organic disease, whereas in secondary/tertiary care, higher cut-off may be recommended to enhance the PPV of the test and thus, to accurately identify patients requiring endoscopic examination.

Concerning stool sampling, watery stool are unsuitable for FC testing. Despite FC is stable in stool up to 1 week at room temperature, it would be advisable to preserve the stool sample at $+4^{\circ}\text{C}$ until delivery in laboratory for analysis. Regarding methods used for FC assessment, a high degree of variability has been widely reported. Since no international standard is available, it is recommended to perform FC monitoring at the same laboratory.

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Table I. Individual, environmental and methodological factors affecting FC levels.

Abbreviations: FC, fecal calprotectin; BMI, body mass index; NSAID, non-steroidal anti-inflammatory drugs; PPI, proton pump inhibitors.

Table II. Comparison of available methods for FC quantitation.

Abbreviations: CLEIA, chemiluminescent enzyme immunoassay; FEC, fecal calprotectin; ELISA, enzyme-linked immunosorbent assay; FEIA, fluorescence enzyme immunoassay; PETIA, particle enhanced turbidimetric immunoassay; POCT, point of care testing.

Figure 1. Bland-Altman plot (A) and Correlation plot (B) of FC levels assessed by Calprest[®] Eurospital and Buhlmann-fCal[®] ELISA assays.

Bland-Altman plot (A) shows the variation of mean FC levels between Calprest[®] Eurospital and Buhlmann-fCal[®] ELISA assays. Correlation plot (B) depicts the deviation of the Passing Bablock regression equation with the corresponding 95% confidence interval respect to the identity line ($y=x$). FC values are reported in $\mu\text{g/g}$.

FC, fecal calprotectin; ELISA, enzyme linked immunosorbent assay.